SHORT COMMUNICATION

Zinc transporter (ZnT)$8_{186-194}$ is an immunodominant CD8$^+$ T cell epitope in HLA-A2$^+$ type 1 diabetic patients

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Abstract

Aims/hypothesis Anti-zinc transporter (ZnT)8 autoantibodies are commonly detected in type 1 diabetic patients. We hypothesised that ZnT8 is also recognised by CD8$^+$ T cells and aimed to identify HLA-A2 (*A*02:01*)-restricted epitope targets.

Methods Candidate epitopes were selected by ZnT8 plasmid DNA immunisation of HLA-A2/DQ8 transgenic mice and tested for T cell recognition in peripheral blood mononuclear cells of type 1 diabetic, type 2 diabetic and healthy participants by IFN-γ enzyme-linked immunospot.

Results White HLA-A2$^+$ adults (83%) and children (60%) with type 1 diabetes displayed ZnT8-reactive CD8$^+$ T cells that recognised a single ZnT8$_{186-194}$ (VAANIVLTV) epitope. This ZnT8$_{186-194}$-reactive fraction accounted for 50% to 53% of total ZnT8-specific CD8$^+$ T cells. Another sequence, ZnT8$_{153-161}$ (VVTGVLVYL), was recognised in

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20% and 25% of type 1 diabetic adults and children, respectively. Both epitopes were type 1 diabetes-specific, being marginally recognised by type 2 diabetic and healthy participants (7-12% for ZnT8$_{186–194}$, 0% for ZnT8$_{153–161}$).

Conclusions/interpretation ZnT8-reactive CD8$^+$ T cells are predominantly directed against the ZnT8$_{186–194}$ epitope and are detected in a majority of type 1 diabetic patients. The exceptional immunodominance of ZnT8$_{186–194}$ may point to common environmental triggers precipitating beta cell autoimmunity.

Keywords Autoimmunity · Beta cell · Environment · Islet · SCL30A8 · Type 1 diabetes

Abbreviations

ELISpot	Enzyme-linked immunospot
IA-2ic	Intracellular part of protein tyrosine phosphatase 2
MAP	*Mycobacterium avium paratuberculosis*
PBMC	Peripheral blood mononuclear cell
PD	Pyruvate dehydrogenase
SFC	Spot-forming cells
ZnT	Zinc transporter

Introduction

The autoimmune cascade leading to beta cell destruction in type 1 diabetes is propagated by T cell recognition of specific epitopes. Epitope mapping is therefore critical for staging of autoimmunity during the disease, and for developing and monitoring immunomodulatory therapies. Indeed, a combination of multiple epitope markers is likely to track beta cell autoimmunity more efficiently than any single epitope. Likewise, restoring immune tolerance to a broad antigen spectrum may be clinically more effective [1].

Zinc transporter (ZnT)8 was recently identified as a major type 1 diabetes antigen, with 60–80% of recent-onset type 1 diabetic participants harbouring antibodies against its C-terminal domain [2]. As for other beta cell antigens, such autoantibodies are likely to be accompanied by autoreactive T cells targeting ZnT8 [1]. Indeed, ZnT8-reactive CD4$^+$ and CD8$^+$ T cells were recently described in white and Chinese type 1 diabetic patients, respectively [3, 4]. Given their central role in beta cell autoimmunity [5, 6], further characterisation of these CD8$^+$ T cells is needed. Here, we identify a highly immunodominant ZnT8$_{186–194}$ epitope recognised by such cells.

Methods

ZnT8 peptide library We synthesised 70 nonamer peptides (>75% purity; ProImmune, Oxford, UK) that had been selected as potential binders to HLA-A1, -A2, -A3, -A24, -B7 and -B8 (electronic supplementary material [ESM] Table 1). Binding to recombinant HLA-A2 molecules was assessed using Class I REVEAL assays (ProImmune). Candidate epitopes were re-synthesised at >90% purity.

DNA immunisation HLA-A2/DQ8 transgenic ($A^*02:01/ DQB1^*03:02$) $H-2D^{b-/-} \beta_2 m^{-/-} IA^b \beta^{-/-} IA^b \alpha^{-/-} IE^b \beta^{-/-}$ mice were obtained by crossing HLA-A2 and HLA-DQ8 transgenic mice [7, 8]. These mice were pretreated with cardiotoxin and vaccinated twice with non-coding plasmid or plasmid encoding full-length human ZnT8 (1:1 mixture of Arg/Trp$_{325}$ isoforms) cloned into pcDNA3.1, as described previously [7]. The study was approved by the local ethics committee. Splenocytes were plated (5×10^5/well) in IFN-γ enzyme-linked immunospot (ELISpot) plates (Millipore, Molsheim, France) along with peptides (10 μmol/l). After culturing for 20 to 24 h, plates were treated with a biotinylated anti-IFN-γ antibody (U-CyTech, Utrecht, the Netherlands), alkaline phosphatase-conjugated streptavidin and NBT-BCIP (Sigma, Lyon, France). Triplicate wells were counted and averaged using a Bioreader 5000 Pro-SF equipment (BioSys, Karben, Germany).

Study participants We recruited the following participant groups in Paris and Turin: (1) new-onset autoantibody-positive type 1 diabetic adults (n=12; 8 men, 4 women; median age 31 years [range 24–60]; diabetes duration 39 days [0–140]) and children (n=10; 6 boys, 4 girls; median age 12 years [1–16]; diabetes duration 5 days [0–80]); (2) type 2 diabetic patients (n=24; 15 men, 9 women; median age 60 years [12–77]; diabetes duration 12 years [0–42]); and (3) healthy controls (n=27; 12 men, 15 women; median age 36 years [22–60]). Serum antibodies were measured by RIA, using ^{35}S-labelled GAD65, the intracellular part of protein tyrosine phosphatase-2 (IA-2ic) and ZnT8 (construct C-term CRCW JH6.2). All participants were HLA-A2$^+$ (HLA-A*02:01) by genotyping and gave written informed consent. Local ethics committees approved the study.

ELISpot assays Peripheral blood mononuclear cells (PBMCs) prepared as described previously [5] were used fresh or frozen-and-thawed with similar results [5, 9]. PBMCs (3×10^5/well) were seeded in anti-IFN-γ-coated ELISpot plates in AIM-V medium (Life Technologies, Saint Aubin, France) containing 0.5 ng/ml IL-7 and 10 μmol/l peptide. A viral peptide mix (Flu MP$_{58–66}$, Epstein–Barr virus BMLF$_{280–288}$, cytomegalovirus pp65$_{495–503}$) and phytohaemagglutinin (1 μg/ml) were used as positive controls. Pyruvate dehydrogenase (PD)$_{5–13}$ and DMSO diluent were negative controls. After 20 to 24 h, plates were developed and counted as above. For blocking experiments, anti-CD8

(OKT8, 25 μg/ml) or anti-HLA-A2 (BB7.2, 50 μg/ml, both in-house) were added to wells. Readouts are expressed as spot-forming cells (SFC)/10^6 PBMCs after subtracting background responses against PD_{5-13} and no peptide (which were identical in all cases). The positive cut-off was set at 3 SD above the average background, as determined by receiver-operator characteristics analysis [5]. Intra- and inter-assay CVs are 14% and 9%, respectively [5].

Statistics Values are expressed as mean±SD or median (range), according to distribution. Comparisons between proportions were made with Fisher's exact test.

Results

We used DNA immunisation [7] to pinpoint naturally processed and presented HLA-A2-restricted epitopes derived from ZnT8 (Fig. 1a). Based on positive responses (i.e. >3 SD above background) in >15% (≥2/12) of immunised mice, ten candidates were selected (Fig. 1b, ESM Table 1). None was positive in control immunised mice. Similar results were obtained using HLA-A2 transgenic mice lacking the DQ8 transgene [7] (data not shown). Most candidate epitopes were located in the fourth transmembrane region (3/10) or in the C-terminal domain (3/10). None overlapped with the Arg/Trp_{325} polymorphic region.

Binding assays to recombinant HLA-A2 molecules were performed in parallel (Fig. 1c). While most (8/10; 80%) candidate epitopes identified by DNA immunisation were strong HLA-A2 binders, two epitopes (number 50, $ZnT8_{253-261}$ and number 57, $ZnT8_{280-288}$) were weak binders. In addition, several epitopes (22/30; 73%) that were strong HLA-A2 binders did not elicit significant responses in DNA-immunised mice. Thus, compared with epitope selection based on HLA-binding affinity, DNA immunisation allowed us to focus on fewer candidates (10/70 vs 30/70; 14% vs 43%), including two weakly binding
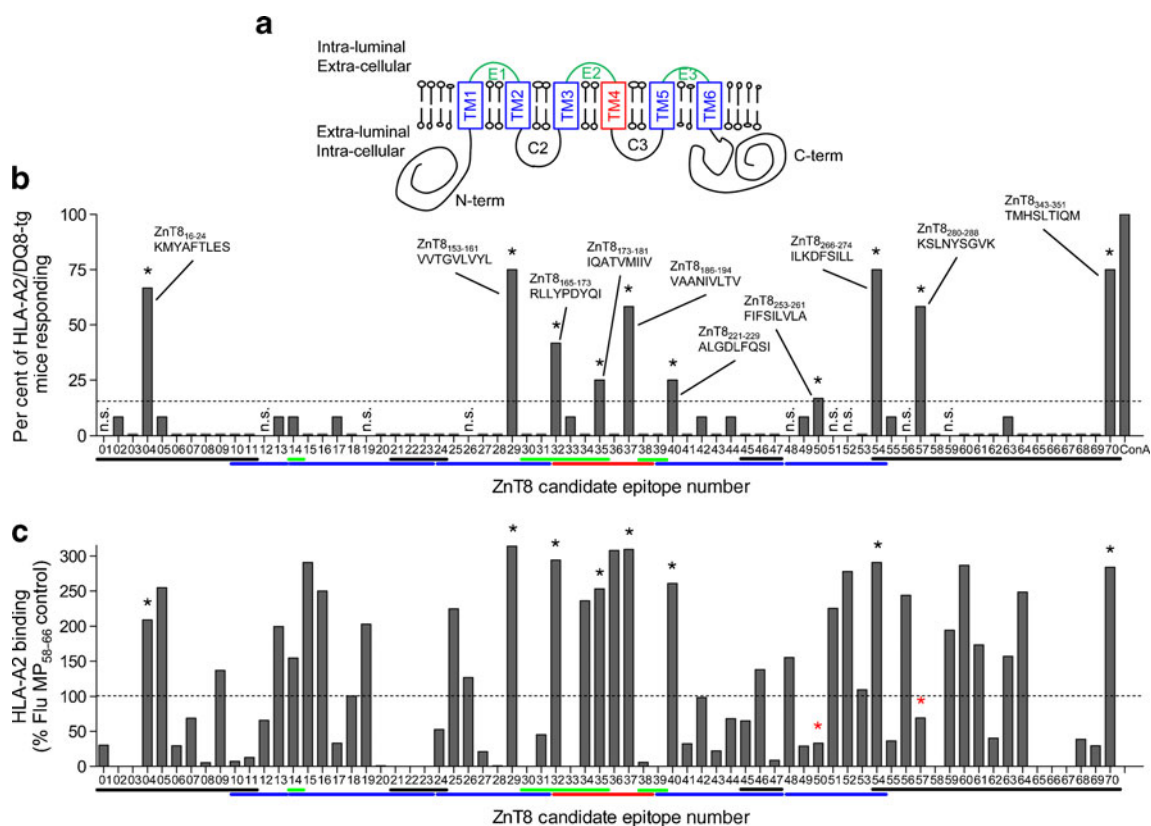

Fig. 1 HLA-A2-restricted ZnT8 candidate epitopes. **a** ZnT8 protein structure and topology. The three intra-luminal loops are shown in green, the four extra-luminal domains in black and the six transmembrane (TM) domains (TM1–TM6) in blue, except TM4 (red). **b** ZnT8 candidate epitopes selected by DNA immunisation in HLA-A2/DQ8 transgenic (tg) mice (*n*=12). Splenocytes were restimulated in vitro with the 70 peptides listed in ESM Table 1. Bars represent the per cent of mice responding to the designated peptide or to concanavalin A positive control. The dotted line represents the positive cut-off (>15% of mice responding). Selected epitopes are indicated by asterisks. Coloured lines (*x*-axis) show the position of each epitope within the ZnT8 structure, according to the colour code above (**a**). **c** Binding of ZnT8 peptides to recombinant HLA-A2.1 in vitro. The same peptide library was tested using the Class I REVEAL binding assay (Proimmune). Results are expressed as per cent binding; the dotted line shows the positive cut-off (>100% binding compared with the reference Flu MP_{58-66} peptide). Asterisks indicate peptides selected by DNA immunisation, two of which (red) are weak binders

peptides. Moreover, DNA immunisation selects candidates on the additional criterion of natural processing and presentation [7].

The identified peptides were subsequently tested for $CD8^+$ T cell recognition using PBMCs from type 1 diabetic, type 2 diabetic and healthy participants. Results are summarised in Fig. 2a and raw data in ESM Figs 1 and 2. All but one peptide ($ZnT8_{221-229}$) were recognised by PBMCs of at least one type 1 diabetic patient. $ZnT8_{153-161}$ (number 29; VVTGVLVYL) and $ZnT8_{186-194}$ (number 37; VAANIVLTV) emerged as immunodominant. $ZnT8_{153-161}$ was recognised in 25% of type 1 diabetic adults and 20% of type 1 diabetic children, but not by type 2 diabetic and healthy participants.

$ZnT8_{186-194}$ was targeted in 73% of type 1 diabetic patients, without significant differences between adults (83%) and children (60%). PBMCs from type 2 diabetic patients and healthy controls rarely recognised this epitope (12% and 7%, respectively; $p \le 0.009$ for both compared with type 1 diabetic adults or children), while $ZnT8_{253-261}$ (number 50; FIFSILVLA) was more frequently recognised in type 2 (25%) than in type 1 diabetic (8% adults, 10% children) and healthy participants (0%; $p=0.007$ for comparison between type 2 diabetic and healthy). $ZnT8_{153-161}$ and $ZnT8_{186-194}$ were strong HLA-A2 binders, were located in transmembrane regions and positive in 75% and 58% of immunised mice, respectively. IFN-γ ELISpot assays
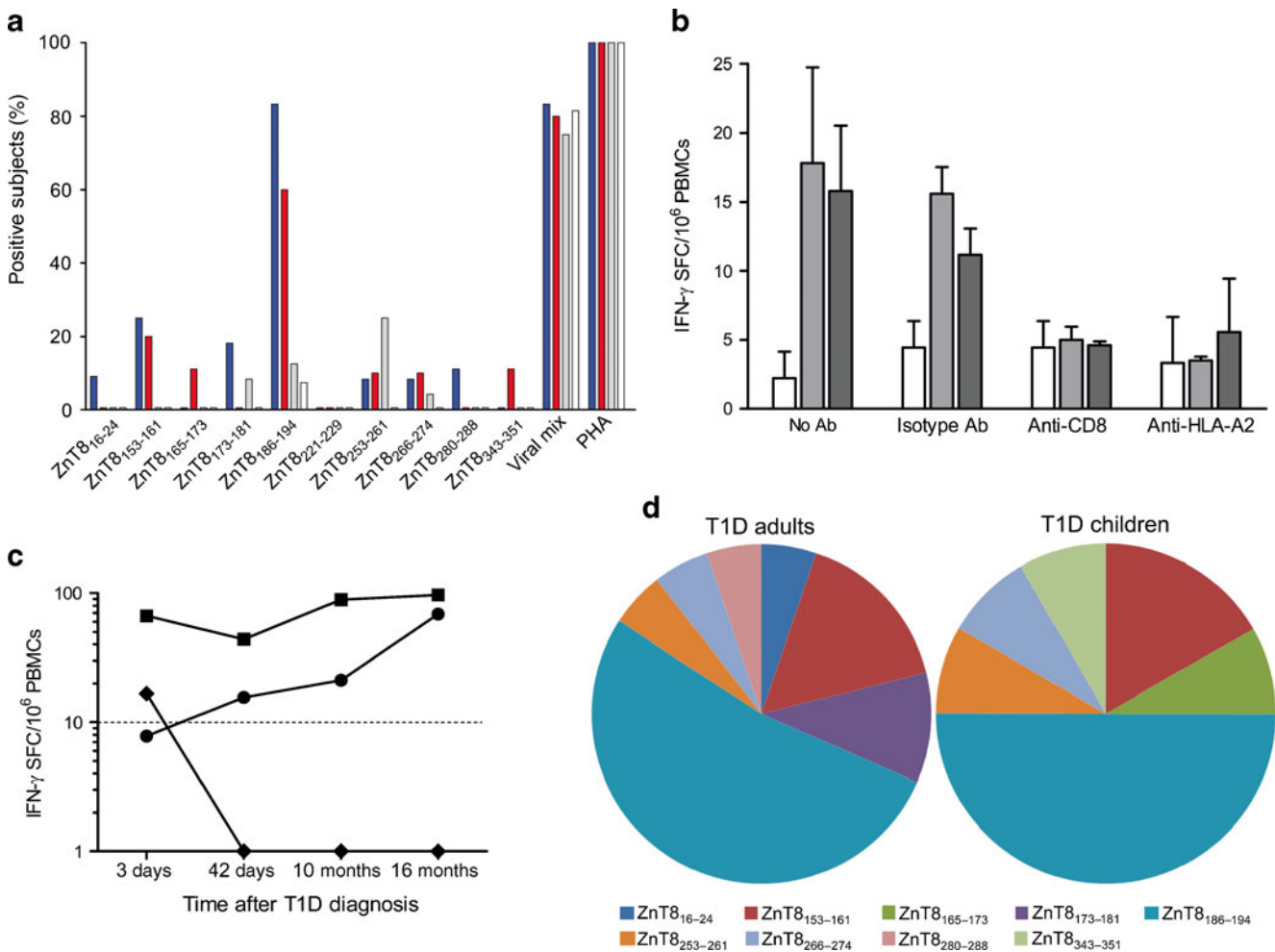
Fig. 2 Recognition of HLA-A2-restricted ZnT8 epitopes in different study participants. **a** Per cent of new-onset type 1 diabetic adults (blue bars; $n=12$) and children (red bars; $n=10$), and of type 2 diabetic (grey bars; $n=24$) and healthy participants (white bars; $n=27$) responding to each individual epitope by IFN-γ ELISpot. **b** IFN-γ ELISpot responses were measured in a type 1 diabetic adult using PBMCs stimulated in the presence of no antibody (Ab), isotype control antibody, anti-CD8 or anti-HLA-A2 antibody. Counts (mean±SD) of triplicate wells are given without background subtraction for each of the indicated peptide stimuli, i.e. no peptide (white bars), $ZnT8_{186-194}$

(light grey bars) and $ZnT8_{253-261}$ (dark grey bars). **c** Patient A03 was tested at four subsequent time-points for PBMC reactivities against the peptides $ZnT8_{186-194}$ (circles) and $ZnT8_{153-161}$ (diamonds), and the viral mix (squares). Results are expressed as mean IFN-γ SFC/10^6 PBMCs and are background-subtracted. **d** Relative distribution of epitope specificities in type 1 diabetic (T1D) adults and children as indicated. The per cent prevalence of each epitope out of all epitopes recognised among new-onset type 1 diabetic adults ($n=19$) and children ($n=12$) is shown

($n=14$) were performed using two different ZnT8$_{186-194}$ peptide batches and yielded identical results in 13 (93%) of 14 assays. Moreover, reactivity to ZnT8 epitopes was neutralised by anti-CD8 and anti-HLA-A2 antibodies (Fig. 2b), demonstrating that IFN-γ responses originated from CD8$^+$ T cells that recognised HLA-A2-restricted epitopes. As previously observed for GAD- and IA-2-reactive CD8$^+$ T cells [5, 7] and for ZnT8-reactive CD4$^+$ T cells [3], there was no correlation between anti-ZnT8 T cell and autoantibody responses (data not shown).

The median frequency of ZnT8 epitope-reactive CD8$^+$ T cells in new-onset type 1 diabetic patients (34.5 SFC/10^6 PBMCs, i.e. 0.003%, range 0.0007–0.08%) was similar to that previously observed for other beta cell epitopes [5, 7]. Moreover, one type 1 diabetic adult patient (identified as A03) was studied at four different time points (Fig. 2c). While IFN-γ responses to a viral mix remained fairly stable over time, frequencies of ZnT8$_{186-194}$-reactive T cells secreting IFN-γ gradually increased over time, while the corresponding ZnT8$_{153-161}$-reactive T cells rapidly disappeared.

The immunodominance of ZnT8$_{186-194}$ was also evident when considering its relative representation among all epitopes recognised (Fig. 2d). Indeed, ZnT8$_{186-194}$ was the most frequently targeted peptide in type 1 diabetic adults and children (53% and 50% of total ZnT8-reactive CD8$^+$ T cell responses, respectively) compared with other epitopes.

Discussion

ZnT8 is clearly a major target of autoreactive CD8$^+$ T cells in white new-onset type 1 diabetic patients. Strikingly, a single HLA-A2-restricted ZnT8$_{186-194}$ epitope was recognised by the overwhelming majority of ZnT8-reactive T cells (50–53%), and in both type 1 diabetic adults (83%) and children (60%). Another epitope, ZnT8$_{153-161}$, ranked second, being recognised in 20% to 25% of type 1 diabetic patients. Such a high degree of epitope focusing is surprising, as autoreactivity is usually more widely distributed against different sequences [5]. Indeed, the two most immunodominant epitopes described to date are GAD$_{114-123}$ [5] and preproinsulin (PPI)$_{15-24}$ [6], which are recognised in ~50% of type 1 diabetic adults compared with the 83% scored by ZnT8$_{186-194}$. An increase in ZnT8$_{186-194}$-reactive IFN-γ responses during the disease was also observed in one patient, contrary to observations for most epitope reactivities, including ZnT8$_{153-161}$, which rapidly wane after diagnosis [9]. ZnT8$_{253-261}$ was recognised in 25% of type 2 diabetic patients, a population seldom included in previous T cell studies. The phenotype of these patients was not distinctive; they were all antibody-negative and all but one not being treated with insulin. However, it is possible that

larger ad hoc studies focusing on patients of uncertain classification may reveal a discrete subset.

The epitopes here identified do not overlap with those targeted by CD4$^+$ T cells [3]. The subdominant ZnT8$_{153-161}$ epitope was also recognised by PBMCs of Chinese HLA-A2$^+$ recent-onset type 1 diabetic patients [4]. In contrast, the novel ZnT8$_{186-194}$ emerges here as the major epitope recognised. Intriguingly, we recently described the same ZnT8$_{186-194}$ epitope and its contiguous ZnT8$_{178-186}$ sequence as targets of autoantibody responses in ~60% of Sardinian type 1 diabetic patients [10]. These autoantibodies were cross-reactive with homologous sequences of the *Mycobacterium avium paratuberculosis* (MAP) 3865c protein, prompting the hypothesis that MAP could be an environmental trigger of type 1 diabetes via a molecular mimicry mechanism [10]. It would therefore be relevant to investigate whether ZnT8$_{186-194}$-reactive CD8$^+$ T cells also cross-recognise MAP3865c$_{133-141}$, which, if confirmed, could explain their high prevalence.

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